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A reporter system that discriminates EF-hand-sensor motifs from signal-modulators at the single-motif level

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ABSTRACT

The T-protein is a single-polypeptide bi-functional enzyme composed of a chorismate mutase domain fused to a prephenate dehydrogenase domain (TyrA). We replaced the chorismate mutase domain with canonical or pseudo-Ca^{2+}-binding motifs (EF-hand). Canonical-EF-hand-motifs differentiate from pseudo-EF-hand-motifs by experimenting a Ca^{2+}-dependent conformational change. The Ca^{2+}-free EF-hand-TyrA fusion-proteins showed TyrA activity at the T-protein level. Canonical-EF-hand-TyrA fusions showed a Ca^{2+}-dependent loss of TyrA activity, but a pseudo-EF-hand-TyrA fusion showed high TyrA activity level in excess-Ca^{2+} conditions. Because TyrA activity exhibits robust changes in response to Ca^{2+}-dependent-EF-hand conformational alterations, TyrA could be a good Ca^{2+}-reporter enzyme. A chimeric canonical/pseudo-EF-hand strategy is proposed to confer pseudo-EF-hand motifs with a Ca^{2+}-dependent conformational change.

1. Introduction

Calcium (Ca^{2+})-binding motifs consist of a 30-residue sequence entity that adopts a helix-loop-helix structure (EF-hand). Usually, EF-hand motifs arrange in pairs that form a stable single-globular domain. Canonical EF-hand motifs bind the ion through a 12-residue loop. Ca^{2+}-binding causes a canonical-EF-hand motif to undergo a structural conformational change (a transition from a closed to an open conformation), which results in the exposure of a hydrophobic protein region [1]. Pseudo-EF-hand motifs present a distinctive 14-residue Ca^{2+}-binding loop. Ca^{2+}-free pseudo-EF-hand motifs show a pre-formed Ca^{2+}-binding loop. Ca^{2+}-loaded pseudo-EF-hand presents minor changes limited to a few residue side-chains [2].

Isolated EF-hand motifs are known to fold into a Ca^{2+}-dependent homo-dimeric four-helix bundle structure. A 34-amino acid peptide of chicken troponin-C containing the third Ca^{2+}-binding site (SCIII) forms a homo-dimer of the Ca^{2+}-binding motif in the presence of excess Ca^{2+}. The SCIII motif binds calcium with strong affinity (at approximately 3 μM) [3]. A 39-amino acid peptide of rabbit skeletal troponin-C containing the fourth Ca^{2+}-binding site (TH2) forms a homo-dimer of the Ca^{2+}-binding motif in the presence of excess Ca^{2+}. TH2 shows a weak affinity for Ca^{2+} (approximately 1 mM) [4]. Linse and co-workers [5] demonstrated that the isolated Ca^{2+}-loaded calbindin D9k N-terminal motif (ClbN) showed a more populated homo-dimeric state in solution than the isolated Ca^{2+}-loaded C-terminal motif (ClbC). The isolated EF-hand motifs showed Ca^{2+} affinities in the 10 to 30 mM range. The same research group also reported that 4 individual EF-hand motifs from calbindin D28k use different mechanisms to achieve the formation of a stable homo-dimeric conformational state [6]. Yang and co-workers [7] recently grafted an isolated EF-hand motif from the stromal interaction molecule-1 (STIM1) into the domain-1 of the adhesion protein CD2 as a scaffold protein. They demonstrated that the isolated EF-hand motif fused to the scaffold protein showed a tendency to homo-dimerize regardless of the Ca^{2+} presence.

Escherichia coli (*E. coli*) prephenate dehydrogenase (TyrA) is a dimeric enzyme that catalyzes the oxidative decarboxylation of prephenate to 4-hydroxyphenylpyruvate using NAD^+ as a cofactor. TyrA and chorismate mutase ($AroQ_T$) form a single-polypeptide bi-functional enzyme known as T-protein. Although experimental and theoretical evidence indicates that the *E. coli* $AroQ_T$ and TyrA domains are interdependent [8,9], we recently described the successful replacement of the $AroQ_T$ domain of the T-protein with the small protein-G a/β immunoglobulin-binding β1 domain (Gβ1) [10]. The $G\beta1_{wt}$-TyrA fusion protein folded into a dimeric conformation and showed such strong TyrA activity that we were able to discard important roles for any interface contact formed between $AroQ_T$ and TyrA.

In this work, we found that the replacement of $AroQ_T$ with EF-hand Ca^{2+}-binding motifs produce a dimeric, functional *E. coli* TyrA protein. Interestingly, we found a calcium-dependent switch-off of

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TyrA activity in constructs containing a canonical-EF-hand motif fused to TyrA. Our work indicates that the Ca^{2+}-dependent switch that controls TyrA activity could be used as a Ca^{2+}-reporter system.

2. Materials and methods

All of the nucleic acid manipulations were performed according to standard procedures [11].

2.1. Bacterial strains and plasmids

E. coli MC1061 Δ*TrpF* was used as the host for all cloning constructs. *E. coli* FA114 [10] was used to over-express the various protein fusion constructs. The pTrc99A plasmid was used as the expression vector. To complete a complementation system for TyrA activity, the gene coding for $AroQ_P$ under a constitutive trc promoter was cloned into the p15A-based plasmid pACYC184, and the final plasmid was transformed to the FA114 bacterial cells.

2.2. Growth media and culture conditions

Cells were cultured in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or in selection medium (based on M9 minimal medium) made as described by Hilvert and co-workers [12], with the appropriate antibiotics. M9 minimal medium contains M9 salts (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl) plus 2 g/L glucose, 1 mM $MgSO_4{\cdot}7H_2O$, and 1 mg/L thiamin–HCl. Antibiotics were used at the following final concentrations: 200 μg/ml ampicillin and 50 μg/ml kanamycin. The M9 minimal medium was supplemented with 0.01 g/L *p*-hydroxybenzoic acid, 0.01 g/L *p*-aminobenzoic acid, 0.01 g/L 2,3-dihydroxybenzoic acid, 0.02 g/L L-tryptophan, 0.02 g/L L-phenylalanine, 0.02 g/L L-leucine, 0.02 g/L L-isoleucine, and 0.1 mM isopropyl-β-thiogalactopyranoside (IPTG).

2.3. Complementation assays

Particular variants were transformed into the FA114 strain. Individual colonies were picked and grown overnight in 1 ml of LB medium supplemented with antibiotics at 30 °C with agitation. Around $5X10^8$ cells were washed three times with around 400 μl of cold 1X M9 salts. 20 μl of the washed cells were used to inoculate 2 ml of liquid complementation media. The culture OD at 600 nm was followed for several days to record the growth rates of the particular clones.

2.4. Construction of TyrA fusion proteins

To fuse the different EF-hand motifs to the N-terminus of TyrA, we used a modified wild-type gene coding for the bifunctional $AroQ_T$-TyrA protein with the substitution C95Q placed near the junction between both domains. This change introduced a *Pst*I restriction site at the gene region coding for the $AroQ_T$-TyrA protein junction. The gene variants were constructed by inverse-PCR mutagenesis. To purify the $TyrA_{wt}$ protein or the different TyrA fusion proteins, a histidine tail was added to each protein N-terminus.

2.5. Bacterial extracts

For the preparation of crude cell extracts, FA114 cells, harboring $tyrA_{Wt}$ or tyrA variants, were grown at 30 °C with agitation (300 rpm) in 15 ml of LB medium supplemented with antibiotics until reaching an OD_{600nm} of 0.6. IPTG was then added (0.5 mM final concentration), and the cultures were incubated at 30 °C for another 5 h. The cells were harvested, and approximately 30 OD_{600nm} of cell paste was resuspended in 300 μl of lysis buffer (0.05 M Tris pH 7.5, 0.1% BSA; no protease-inhibitor cocktail or PMSF was included in the buffer). Cells were disrupted by two rounds of 25-s sonication pulses (Branson Sonifier, Danbury, CT, USA). The cellular debris were removed by centrifugation for 10 min at room temperature, and the supernatant (bacterial extract) was collected to measure TyrA activity as described below.

2.6. Protein fusion over-expression

FA114 cells, harboring $tyrA_{Wt}$ or tyrA variants, were grown at 30 °C with agitation (300 rpm) in 100 ml of LB medium supplemented with antibiotics until reaching an OD_{600nm} of 0.6. IPTG was then added (0.5 mM final concentration), and the cultures were incubated at 30 °C for an additional 4 h.

2.7. Protein purification and size-exclusion chromatography

Cell pellets were resuspended in 5 ml of 50 mM Tris buffer, pH 7, 500 mM NaCl, 5% glycerol, 5 mM of imidazole, lysed by sonication (Branson Sonifier 450; 20 s, six times in 30-s intervals, 50% pulse, 4 °C) and centrifuged for 20 min at 12000 rpm at 4 °C. Variants were purified from the soluble cell fraction by loading the cell extract into a 1-ml nickel–sepharose column previously equilibrated with the sonication buffer. Then, the columns were washed with 10 ml of 50 mM Tris buffer pH 7, 500 mM NaCl and 35 mM imidazole. The bound His6-tagged protein was eluted with 300 mM imidazole in 50 mM Tris buffer pH 7 with 500 mM NaCl. Protein concentrations were measured using the Bradford method [13] with the BIO-RAD protein assay and BSA as the standard protein.

To determine the protein sample oligomerization state, the purified proteins were analyzed by size-exclusion chromatography in an Äkta FPLC system equipped with a UV detector and a size-exclusion Superose HR12 column from Amersham Biosciences (Uppsala, Sweden). The samples were eluted with 50 mM Tris buffer pH 7, 20 mM NaCl at a flow rate of 0.5 ml/min. The protein sample's apparent molecular mass was determined by comparing the protein elution volume with purified BSA as MW standard. Approximately, 40–50 μg of pure protein was injected to the FPLC system.

2.8. TyrA activity assays

TyrA activity was measured as described [14]. TyrA activity was determined by monitoring the formation of NADH at 340 nm in 96-well plates using a Safire plate reader controlled by a Tecan platform (Tecan Group LTD, Männedorf, Switzerland). The reaction mixtures (150 μl) contained 0.4 mg of purified protein in 50 mM Tris, 0.2 mg/ml bovine serum albumin (BSA), and 2 mM NAD^+. The reaction was initiated by the addition of prephenate (PPA) (~0.2 mM final concentration). When indicated, EDTA (1 mM final concentration) or calcium chloride (2 mM final concentration) was added to the reaction mixture.

3. Results and discussion

3.1. The $AroQ_T$ domain of the T-protein can be replaced with EF-hand motifs

We recently proposed a structural role for the $AroQ_T$ domain in T-protein function [10]. We discovered that the dimeric nature of $AroQ_T$ is more important for stabilizing the dimeric TyrA domain than any specific contact interface formed between both protein molecules. Alternative protein domains can replace the $AroQ_T$ domain of the T-protein only if the new partner successfully mimics the structural role played by the $AroQ_T$ domain in the T-protein.

As described in the Introduction section, isolated or grafted EF-hand motifs tend to adopt a homo-dimeric structure. Therefore, we reasoned that individual EF-hand motifs could be good candidates to replace the $AroQ_T$ domain of the T-protein.

In Fig. 1, we show individual EF-hand motifs fused to the N-terminus of TyrA. We included the above-described EF-hand III (SCIII) and IV (TH2) of troponin C and the ClbN (pseudo) and ClbC (canonical) EF-hand motifs present in calbindin D9k.

As shown in Fig. 2, the T-protein TyrA domain was fully active independently of the presence of EDTA or Ca^{2+} in the reaction buffer. The low in-vivo Ca^{2+} concentration in bacteria (0.1–0.3 μM) [15] together with a weak Ca^{2+}-affinity EF-hand motif (1 mM for the TH2 EF-hand motif) suggests a free-Ca^{2+} form of the purified TH2-TyrA fusion protein. As expected, adding EDTA to the reaction buffer had no effect, but the addition of Ca^{2+} inhibited TyrA activity. On the other hand, the strong Ca^{2+} affinity of the SCIII EF-hand motif (3 μM) suggests a partial Ca^{2+}-loaded form of the purified SCIII-TyrA fusion protein explaining the partially inhibited TyrA activity with this construct. Removal of the ion with EDTA enhanced TyrA activity, and the addition of Ca^{2+} resulted in nearly complete inhibition of TyrA activity.

The fusion protein ClbC-TyrA showed low TyrA activity levels. Nevertheless, the canonical EF-hand motif still confers a Ca^{2+}-dependent switch that controls TyrA activity (Fig. 2). The poor-tendency of the ClbC canonical-EF-hand motif to adopt a dimeric conformation in isolation [5] could explain the result.

The Ca^{2+}-free ClbN pseudo-EF-hand is known to have a preformed Ca^{2+}-binding site [2]. In the Ca^{2+}-bound state, the pseudo-EF-hand motif remains essentially intact, showing only some side-chain reorganization [2]. Thus, minor perturbations in TyrA activity were observed with the purified ClbN-TyrA fusion protein in the presence of excess Ca^{2+} (Fig. 2).

Our results clearly show that in a Ca^{2+}-free state, both canonical- or pseudo-EF-hand motifs allow TyrA function. Ca^{2+}-loaded canonical-EF-hands somehow interfere with TyrA activity. Since Ca^{2+}-loaded canonical EF-hand motifs rearrange from a closed to an open conformation, it is tempting to speculate that this conformational rearrangement could perturbs TyrA activity.

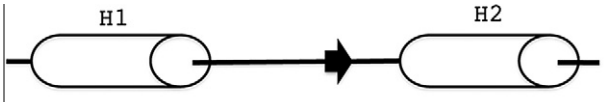
3.2. Impairing ion binding eliminates the Ca^{2+}-dependent TyrA activity switch

Chazin and coworkers [16] reported the characterization of the calbindin D9k mutant E65Q. E65 is located in the ClbC Ca^{2+}-binding loop and provides two important ion contacts. Thus, the substitution to glutamine was shown to severely affect the Ca^{2+} affinity of the C-terminal EF-hand. We introduced the E65Q substitution in the isolated ClbC EF-hand motif. The purified $ClbC_{E65Q}$-TyrA fusion protein showed strong dehydrogenase activity independently of the presence of EDTA or Ca^{2+} (Fig. 2). Therefore, ion-binding impairment in the canonical EF-hand motif resulted in a fully active TyrA protein even in the presence of excess Ca^{2+}.

3.3. The Ca^{2+}-dependent TyrA activity switch tolerates some substitutions in the EF-hand Ca^{2+}-binding loop

Inspection of the amino acid sequence of the troponin C SCIII EF-hand motif identified three Ca^{2+}-binding loop residues that slightly deviate from the canonical residues expected at such positions [1]. We constructed and fused to TyrA, a triple-substituted SCIII variant ($SCIII_{cons}$), containing the expected consensus residues at such positions (see Fig. 1). As shown in Fig. 2, the strong TyrA activity of the $SCIII_{cons}$-TyrA fusion protein suggests a free-Ca^{2+} form for the purified protein. A weaker Ca^{2+}-affinity for the $SCIII_{cons}$ EF-hand (as a result of the three amino acid substitutions) compared to the unmodified SCIII motif could explain the obtained results. As expected, adding EDTA to the reaction mixture did not modify the strong TyrA activity. However, the triply substituted SCIII motif showed a strong Ca^{2+}-dependent switch that controlled TyrA activity.

Deviations in the strongly conserved residue positions had been previously observed in canonical-Ca^{2+}-binding loops [1]. By monitoring TyrA activity in the presence of Ca^{2+}, it might be possible to use this reporter system to systematically construct a family of Ca^{2+}-binding loop amino acid sequences still compatible with ion-binding and showing a Ca^{2+}-dependent perturbation of TyrA function. Identification of functional canonical-like Ca^{2+}-binding loop members will help to clarify the roles that conserved and variable amino acid residues could play in Ca^{2+}-binding and in the Ca^{2+}-dependent structural transition. Furthermore, we stated above that TyrA fused to a canonical-EF-hand-sensor motif is inactive in the presence of excess Ca^{2+}. Due to the low bacterial intracellular Ca^{2+} level, in order to have an in vivo TyrA-inactive state, the EF-hand-sensor-like motif must contain a Ca^{2+}-binding loop with strong ion-binding properties. We propose that the use of this reporter system (growing mixed population of cells under different selection protocols) and the quantification (using high-throughput sequencing) of the enrichment or depletion [17] of the different EF-hand-TyrA variants over culture time could help to identify canonical-like EF-hand motifs containing Ca^{2+}-binding loops with strong ion-binding affinities.

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DEEIESLMKDG    DKNNDGRID    FDEFLKMMEGVK    TH2
KSEEELANAFRIF  DKNADGYID    IEELGEILRATG    SCIII
** *** . *. :                *** :*::
KSPEELKGIFEKY  AAKEGDPNQLS  KEELKLLLQTE     ClbN
STLDELFEEL     DKNGDGEVS    FEEFQVLVKKISQ   ClbC
KSPEELKGIFEKY  DKNADGYID    IEELGEILRATG    H1ClbN-SCIII
KSPEELKGIFEKY  DKNADGYID    KEELKLLLQTE     H1H2ClbN-SCIII
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Fig. 1. Amino acid sequences of Ca^{2+}-binding motifs fused to TyrA. The underlined residues in the SCIII Ca^{2+}-binding loop indicate amino acid residues that slightly deviate from the expected consensus amino acids at those positions. Helices H1 and H2 from SCIII and ClbN show a strong sequence conserved pattern (approximately 40% identity and 60% similarity). The underlined residues in the bovine ClbN H2 helix indicate charged residues that are replaced with uncharged residues in the SCIII H2 helix. The underlined glutamate residue in the bovine ClbC H2 helix was replaced with a glutamine residue as described in the text. The underlined hydrophobic residue indicated in TH2, SCIII, ClbN and ClbC is found at the protein core of the EF-hand four-helix bundle. The underlined glutamate residue in the bovine ClbN H2 helix was replaced with an arginine residue as described in the text. The H1ClbN-SCIII EF-hand motif contains the SCIII H1 helix replaced by the H1 helix present in the bovine ClbN motif. The H1H2ClbN-SCIII EF-hand motif contains the SCIII H1 and H2 helices replaced by the H1 and H2 helices present in the bovine ClbN motif.

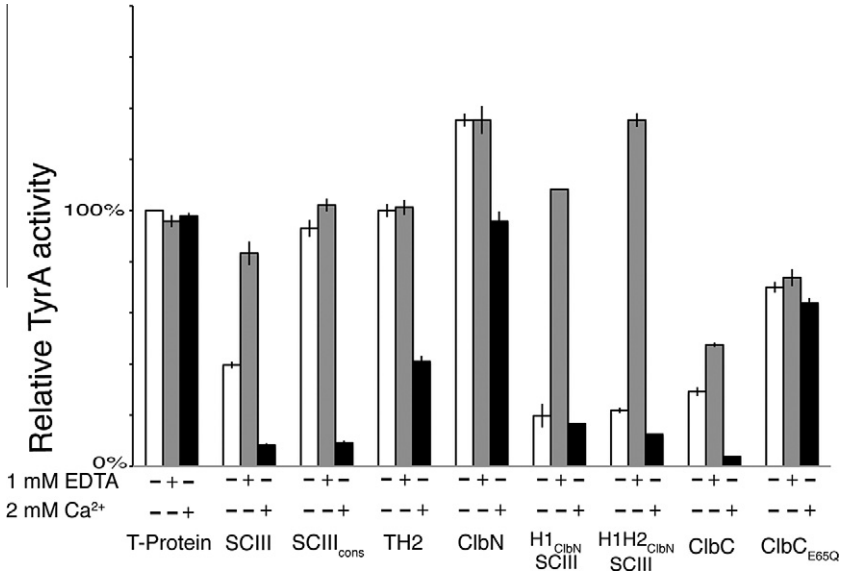

Fig. 2. Comparison of the TyrA activity of the indicated purified fusion proteins and purified T-protein. White bars indicate TyrA activity measurements in the absence of EDTA or Ca^{2+}. Gray bars indicate TyrA activity measurements in the presence of 1 mM EDTA. Black bars indicate TyrA activity measurements in the presence of 2 mM Ca^{2+}. TyrA activity at 100% was defined as 0.96 μmol of NADH produced per second per milligram of purified T-protein.

3.4. Introducing a Ca^{2+}-dependent control-switch to TyrA fused to a pseudo-EF-hand motif

Drakenberg and coworkers [18] reported the successful conversion of the ClbN pseudo-EF-hand Ca^{2+}-binding loop into a functional canonical-like Ca^{2+}-binding loop. The complete calbindin D9k molecule containing the designed Ca^{2+}-binding loop did not show the expected Ca^{2+}-induced conformational change. Chazin and coworkers [19] recently designed calbindomodulin (CBM-1). The approach to design CBM-1 consisted of introducing amino acid substitutions predicted to destabilize the calbindin D9k closed conformation and stabilize for calbindin D9k open conformation. It was shown that CBM-1 undergoes a Ca^{2+}-induced conformational transition from a closed to an open conformation that results in the solvent exposure of a hydrophobic region.

We described above that Ca^{2+}-loaded pseudo-EF-hands fused to TyrA do not interfere with TyrA activity. To introduce a Ca^{2+}-dependent switch to control TyrA activity in a pseudo-EF-hand-TyrA fusion protein, we utilized the following approach: first, we identified a large patch of conserved amino-acid sequence in the H1 and H2 helices present in the troponin C EF-hand III (SCIII, a canonical-EF-hand) and in bovine ClbN motif (a pseudo-EF-hand) (Fig. 1). The salient differences between the SCIII and ClbN EF-hand motifs consist in the Ca^{2+}-binding loops and in a group of charged residues present in the H2 helix from the pseudo-EF-hand that were replaced with uncharged amino acid residues in the canonical-type EF-hand. Then, we constructed chimeric EF-hand motifs that contain the H1 helix or the H1 plus the H2 helices from the ClbN motif replacing the ones present in the canonical SCIII motif (Fig. 1).

The purified H1ClbN-SCIII-TyrA and H1H2ClbN-SCIII-TyrA fusion proteins showed low TyrA activity levels (Fig. 2). Adding EDTA to the reaction mixture increased the TyrA activity above T-protein levels. The chimeric constructs showed a strong Ca^{2+}-dependent switch that blocked TyrA activity (Fig. 2). Size-exclusion chromatography revealed an important Ca^{2+}-dependent transition of a mostly dimeric conformation to monomeric and higher oligomeric conformations of both fusion proteins (Fig. 3b). This Ca^{2+}-dependent structural transition was not observed with the purified ClbN-TyrA or SCIII-TyrA fusion proteins (Fig. 3a). Excluding the Ca^{2+}-binding-impaired ClbC motif fused to TyrA, the T-protein, and the BSA protein, all protein fusions exposed to Ca^{2+} show an apparent transition from a mostly dimeric conformation to a monomeric conformation (Fig. 3a).

Our results indicate that replacing the 14-residue pseudo-Ca^{2+}-binding loop from ClbN with the 12-residue canonical-Ca^{2+}-binding loop from the SCIII EF-hand motif is sufficient to confer a Ca^{2+}-dependent control of TyrA fused to a pseudo-EF-hand motif.

Drakenberg and coworkers did not observe a Ca^{2+}-dependent conformational change in the calbindin molecule containing their designed ClbN motif [18]. Our designed chimeric H1H2ClbN-SCIII EF-hand motif contains a different canonical-Ca^{2+}-binding loop as compared with the Drakenberg and coworkers design [18]. Furthermore, we fused the isolated chimeric H1H2ClbN-SCIII EF-hand motif to the TyrA protein whereas Drakenberg and coworkers inserted their ClbN motif design back to the calbindin D9k molecule [18]. These differences could be partly responsible of the observed Ca^{2+}-dependent switch that controls TyrA activity when TyrA is fused to the H1H2ClbN-SCIII motif. The Ca^{2+}-dependent switch that controls TyrA activity is absent when TyrA is fused to the ClbN pseudo-EF-hand motif. It will be interesting to construct the Drakenberg-like ClbN design fused to TyrA to test the relevance of the Ca^{2+}-binding loop in the Ca^{2+}-dependent conformational change of isolated EF-hands motifs.

3.5. TyrA as a new Ca^{2+}-sensor protein

GFP-based Ca^{2+}-sensors have been previously described [20–25]. The most successful ones are composed of a calmodulin-binding peptide and a calmodulin molecule (calmodulin contains 4 Ca^{2+}-binding motifs) fused to a circularly permuted GFP molecule [22,23]. The Ca^{2+}-induced interaction between calmodulin and its target peptide alters the emission fluorescence of GFP. Linse and co-workers [24] and Yang and co-workers [25] reported GFP reconstitution (development of fluorescence) from two non-fluorescent GFP fragments by the Ca^{2+}-dependent assembly of two calbindin fragments (residues 1–43 and residues 44–75).

A significative difference between our proposal and the GFP-based reporter systems consists in the ability of our system to monitor a putative Ca^{2+}-dependent conformational change of indi-

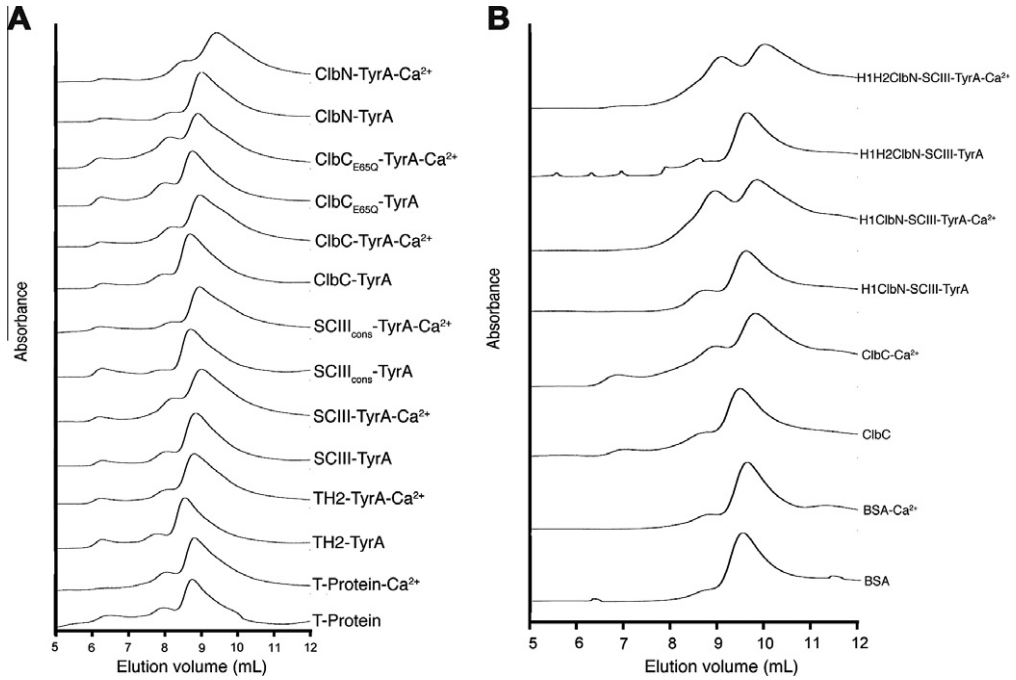

Fig. 3. Analytical gel-filtration chromatograms of the indicated fusion variants and T-protein. A total of 40–50 μg of pure protein was injected into the column. (a) The samples were run at 1 mL per min of running buffer. (b) The samples were run at 0.75 mL per min of running buffer. When indicated, 2 mM calcium chloride was included into the running buffer otherwise the samples contained 1 mM EDTA. Bovine serum albumin (66 kDa) was used as MW standard.

vidual EF-hand motifs. This possibility will open up alternative ways to identify the amino acid residues that control the single-motif conformational response to Ca^{2+}-binding. Our system is different too from the one described by Yang and co-workers [7]. They described a grafting approach of isolated EF-hand motifs or isolated EF-hand loops into the domain-1 of the adhesion protein CD2 as a scaffold protein. Yang and co-workers system is useful to study site-specific information about the EF-hand Ca^{2+} affinity independently of the Ca^{2+}-dependent conformational change.

The proposal we are describing in the present work is easy to implement as demonstrated by the results obtained using crude bacterial cell extracts (Supplementary Fig 1). Measurements of TyrA residual activity (without taking care of the differences in protein expression levels) is enough to find out (1) those EF-hand motifs with poor-Ca^{2+}-affinity that are still able to show a Ca^{2+}-dependent conformational change that perturbs TyrA function (TH2-TyrA (1 mM of Ca^{2+} affinity) and $SCIII_{cons}$-TyrA (unknown Ca^{2+} affinity) fusion proteins in Supplementary Fig 1; these variants did not show a clear gain in TyrA residual activity in the presence of EDTA, therefore, their Ca^{2+}-affinity is well over the 0.1–0.3 μM Ca2+ concentration range inside the bacterial cytoplasm); (2) those EF-hand motifs that lack the Ca^{2+}-dependent conformational change that perturbs TyrA function ($ClbC_{E65Q}$-TyrA and ClbN-TyrA fusion proteins in Supplementary Fig 1 and Fig 1, respectively; these variants show similar TyrA residual activity regarding the presence of excess EDTA or excess Ca^{2+}); (3) those EF-hand motifs showing an intermediate Ca^{2+}-affinity that are still able to show a Ca^{2+}-dependent conformational change that perturbs TyrA function (expected Ca^{2+} affinities should be above the Ca^{2+} concentration range inside the bacterial cytoplasm; SCIII-TyrA (3 μM of Ca^{2+} affinity) and ClbC-TyrA (around 20 μM of Ca^{2+} affinity) fusion proteins in Supplementary Fig 1; these variants show a clear gain in TyrA residual activity in the presence of EDTA and a strong Ca^{2+}-dependent conformational change that perturbs TyrA function); (4) and more importantly, the system is able to find out those EF-hand motifs showing Ca^{2+} affinities in the 0.1–0.3 μM Ca^{2+} concentration range or better that are still able to show a Ca^{2+}-dependent conformational change that perturbs TyrA function (H1H2ClbNSCIII-TyrA fusion protein in Supplementary Fig 1; these variants show an impressive gain in TyrA residual activity in the presence of EDTA. Excess Ca^{2+} do not alter the already perturbed TyrA function).

The fusion protein ClbN-TyrA was the only one that showed unexpected differences when comparing the results obtained using crude bacterial extracts (Supplementary Fig. 1) with the results obtained using the purified fusion variant (Fig 2).

Our Ca^{2+}-reporter approach could be easily implemented as a selection system. We carried out a L-tyrosine deficiency complementation experiment with some of our constructions (Supplementary Fig 2). The results clearly show a delayed complementation phenotype for the H1H2ClbNSCIII-TyrA fusion variant (open-triangles complementation curve in Supplementary Fig 2) as compared with the parent fusion protein ClbN-TyrA (open-squares complementation curve in Supplementary Fig 2) or with a fusion variant showing poor Ca^{2+}-binding affinity (TH2-TyrA fusion variant; open-diamonds complementation curve in Supplementary Fig 2). As a negative control, we included a construction containing a Leucine to Arginine substitution in an important position expected to stabilize the homo-dimeric EF-hand four-helix bundle [3] ($ClbN_{L28R}$-TyrA fusion variant; cross-symbol complementation curve in Supplementary Fig 2). Complementation experiments using solid medium showed clear differences in colony size among the tested variants (we observed very small colonies of bacteria transformed with the plasmid carrying the gene coding for the H1H2ClbNSCIII-TyrA variant as compared to normal-sized colonies formed from bacteria transformed with plasmids carrying the genes coding for the TH2-TyrA or ClbN-TyrA variants; bacteria transformed with the plasmid carrying the gene coding for the $ClbN_{L28R}$-TyrA fusion variant do not formed colonies at all; data not shown).

It will be interesting to characterize the Ca^{2+}-binding affinity of the isolated H1H2ClbNSC3 chimeric EF-hand motif. Experiments aimed to replace the wild-type calbindin ClbN motif with our chimeric design are in progress.

4. Conclusions

In this work, we demonstrate that individual Ca^{2+}-binding motifs are able to stabilize the dimeric TyrA protein. Our results suggest that a putative Ca^{2+}-free homodimeric four-helix bundle conformation adopted by a pseudo-EF-hand or by a canonical-EF-hand motif in the TyrA fusion context could be responsible for permitting the functional dimeric conformation of TyrA.

Ca^{2+}-loaded canonical-EF-hand motifs are known to undergo a conformational transition from a closed to an open state that results in the opening of the four-helix bundle conformation and the solvent exposure of a hydrophobic region. We believe that this Ca^{2+}-dependent conformational transition could perturb TyrA activity. Impairing the canonical-EF-hand ion-recognition process results in a fully activated TyrA protein even in the presence of excess Ca^{2+}. Impairing the EF-hand 4-helix bundle stability completely abolished TyrA function. Alterations in the 12-residue canonical-Ca^{2+}-binding loop still allowed a Ca^{2+}-dependent switch that controlled TyrA function.

We also demonstrated that the construction of chimeric helix-loop-helix Ca^{2+}-binding motifs succeeded to confer a pseudo-EF-hand motif fused to TyrA with a canonical-EF-hand-like Ca^{2+}-dependent switch that controlled TyrA function.

Finally, this work describes a simple-to-construct protein domain combination that produces a non-natural multi-domain protein with entirely new selectable properties. The new multi-domain protein does not require any additional modification to show robust Ca^{2+}-dependent functional regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.07.053.

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